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Improved Method for the Routine Identification of Toxigenic *Escherichia coli* by DNA Amplification of a Conserved Region of the Heat-Labile Toxin A Subunit

T. VICTOR,[1]* R. DU TOIT,[1] J. VAN ZYL,[2] A. J. BESTER,[1] AND P. D. VAN HELDEN[1]

MRC Centre for Molecular and Cellular Biology, Department of Medical Physiology and Biochemistry, University of Stellenbosch Medical School, P.O. Box 63,[1] and Department of Microbiology, Tygerberg Hospital,[2] Tygerberg 7505, Republic of South Africa

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This report describes a DNA amplification procedure for routine identification of heat-labile-toxin-producing *Escherichia coli*. Two oligonucleotide primers were used in a polymerase chain reaction procedure to amplify a highly conserved region of the A subunit of the heat-labile enterotoxin gene. Amplifications were done directly on *E. coli* colonies from plates when *Salmonella*, *Shigella*, or parasite infections were excluded as agents of the severe diarrhea in the patients. The conditions for the polymerase chain reaction method were empirically determined, and the procedure is inexpensive, sensitive, and specific. Positive results can be obtained over a wide variation in bacterial numbers, with no inhibition of *Thermus aquaticus* DNA polymerase. Detection of the amplified product can be done by agarose gel electrophoresis, which is specific and sensitive enough for routine diagnosis of this pathogen in clinical isolates. If greater sensitivity and specificity are required, hybridization with ^{32}P- or alkaline phosphatase-labeled oligonucleotide probes can be used. Our results suggest that heat-labile-toxin-producing *E. coli* is responsible for about 9% of nondiagnosed diarrhea cases in Tygerberg Hospital, Tygerberg, Republic of South Africa.

Serological typing and tissue culture techniques currently used for detection of enterotoxigenic *Escherichia coli* (9, 14, 17) are nonspecific and insensitive and, thus, not used for routine diagnosis. One of the main reasons for this is that the genes that code for these enterotoxins occur on plasmids (18) and enterotoxin production is therefore not strain specific. Enterotoxins produced may be of the heat-labile (LT) or heat-stable (ST) type (10, 18), and *E. coli* may harbor one only or both types simultaneously.

A number of workers have used molecular genetic techniques to assay for the presence of these genes in *E. coli*. Cloned DNA probes and oligonucleotides have been used successfully in colony hybridization for LT and ST toxins, but these methods, although specific, lack sensitivity and require the use of radiolabeled probes and a fairly high degree of sophistication (6, 10, 11, 13, 16). Recently, the genes that code for subunits A and B of LT enterotoxin have been cloned and sequenced (18, 19). This information has made possible the identification of LT toxin-producing *E. coli* from clinical specimens by using the polymerase chain reaction (PCR) technique after extraction of the DNA (3, 12) and amplification of the B subunit of the LT toxin gene (12).

In this report, we describe some improvements to this approach and successful amplification of the A subunit of the LT toxin gene directly, without prior DNA extraction. We have optimized the amplification conditions, and the procedure is inexpensive, sensitive, and simple. Detection of the amplified product can be done by agarose gel electrophoresis, which is specific for the gene of LT enterotoxin and sensitive enough for detection of this pathogen in clinical isolates. We also show that should greater sensitivity or specificity be required, hybridization with a ^{32}P- or alkaline phosphatase-labeled oligonucleotide probe can be done. This procedure is not necessary for routine diagnosis; however, it confirms the accuracy and specificity of our routine gel diagnosis technique.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains. LT (LT$^+$) and ST (ST$^+$) enterotoxin-producing *E. coli* (ATCC 43886 and ATCC 43896, respectively) and positively identified *Shigella flexneri*, *Shigella dysenteriae*, *Salmonella typhi*, *Salmonella typhimurium*, and *Klebsiella pneumoniae* were used in this study.

Selection of clinical isolates for amplification. Stool specimens from patients (mainly infants) with severe diarrhea were received in the diagnostic microbiology laboratory at Tygerberg Hospital, near Cape Town, Republic of South Africa. Samples were routinely plated on MacConkey plates and screened for any *Salmonella*, *Shigella*, or parasite infection. In samples in which no pathogen was detected, a scrape of the thicker part of the mixed growth on the MacConkey plate was taken and thoroughly mixed in 400 μl of sterile saline. Ten microliters of this mixture was used for assay, involving amplification of the LT enterotoxin gene as described below. The same procedure was followed for the control bacterial samples.

Synthetic oligonucleotides. The sequences of the oligonucleotides used are given in Fig. 1. Primers (LT51 and LT31) and probe PLT1 were synthesized on a Beckman System I Plus DNA synthesizer. Probe PLT1 was 5′ end labeled with [^{32}P]ATP (Amersham; specific activity, 5,000 Ci/mmol; 1 Ci = 37 BGq) and T4 polynucleotide kinase (Amersham) and subsequently purified on a 10% polyacrylamide–7 M urea sequencing gel. Probe PLT2 was labeled with alkaline phosphatase at the 5′ end via a C6 amino linker arm (Genetic Designs).

Amplification of the LT enterotoxin gene. Primers were designed to amplify a 110-bp fragment of a conserved region

LT51	CCG GTA TTA CAG AAA TCT GA
LT31	GTG CAT GAT GAA TCC AGG GT
PLT1	TAG CAG GTT TCC CAC CGG AT
PLT2	TAG CAG GTT TCC CAC CGG AT

FIG. 1. Sequences of oligonucleotide probes and primers (sequences are from 5′ to 3′).

of the *toxA* (A subunit) gene (18). For amplification, 10 μl of the saline suspension of bacteria was used and combined in a total volume of 99 μl with a premixture of 1× PCR buffer (70 mM Tris hydrochloride [pH 8.8], 2 mM $MgCl_2$, 0.1% Triton X-100); a 200 μM final concentration (each) of dATP, dGTP, dCTP, and dTTP; and each primer at 0.4 μM. The reaction mixtures were heated initially to 95°C for 10 min to lyse the bacteria and denature the DNA. This was followed by centrifugation in an Eppendorf desktop centrifuge (15 s) and addition of 0.5 U of *Thermus aquaticus* DNA polymerase (New England BioLabs). The mixture was overlaid with 40 μl of mineral oil, and the heating cycle of 93°C for 1 min, 55°C for 1 min, and 72°C for 2 min was repeated 35 times in a thermal cycler.

Detection and analysis of the amplified product. Detection of PCR products was done by electrophoresis on a 1.7% agarose gel (a 20-μl sample) or after dot blot hybridization. In this situation, a 20-μl sample of each PCR mixture was adjusted to 0.4 N NaOH–24 mM EDTA in a 200-μl final volume, applied to a Hybond N nylon filter (Amersham) in a dot blot apparatus (4), and cross-linked on a transilluminator for 3 min. Each filter was prehybridized in 10 ml of 5× SSPE (1× SSPE is 10 mM sodium phosphate [pH 7.0], 0.18 M NaCl, and 1 mM EDTA)–0.5% sodium dodecyl sulfate (SDS)–5× Denhardt solution (1× Denhardt solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin) for 30 min at 54°C. Approximately 1 pmol of ^{32}P-labeled probe PLT1 was then added and hybridized at 54°C for 1 h. Filters were washed twice in 2× SSPE–0.1% SDS for 30 min at room temperature and then for 10 min in 5× SSPE–0.1% SDS at 64°C. The filters were then autoradiographed at −70°C for 2 to 18 h. The same conditions were used for hybridization with the nonradioactive probe; however, detection was done as described elsewhere (2).

RESULTS

Specificity. Control bacterial strains and mixtures of positive and negative strains were directly amplified from saline suspensions by using LT enterotoxin-specific primers. Agarose gel electrophoresis of the samples subjected to thermal cycling detected a product of approximately 110 bp in all positive controls, but no such product was seen in negative samples (Fig. 2). Amplified DNA fragments of approximately 40 bp were seen in all of the samples tested. These fragments are primer dimers, a well-known artifact of many PCRs (5). Faint high-molecular-weight fragments were detected in some *Shigella* samples, but these were nonspecific and could not be confused with the enterotoxin gene fragments on either gel electrophoresis or hybridization (Fig. 2).

As a check that the 110-bp product was the *toxA* gene fragment, dot blot hybridization to the ^{32}P- or alkaline phosphatase-labeled probe (probe PLT1 or PLT2, respectively) was done. Positive signals were obtained for the LT$^+$ *E. coli* samples, but no signal was obtained for the negative strains (Fig. 2). To exclude the possibility of reagent contamination, a control containing saline only instead of template DNA was included in each experiment (Fig. 2). Other negative control strains (see Bacterial Strains) also gave a negative signal (data not shown). These results indicate that the LT gene can be amplified without prior DNA extraction and that the method is specific for the LT gene.

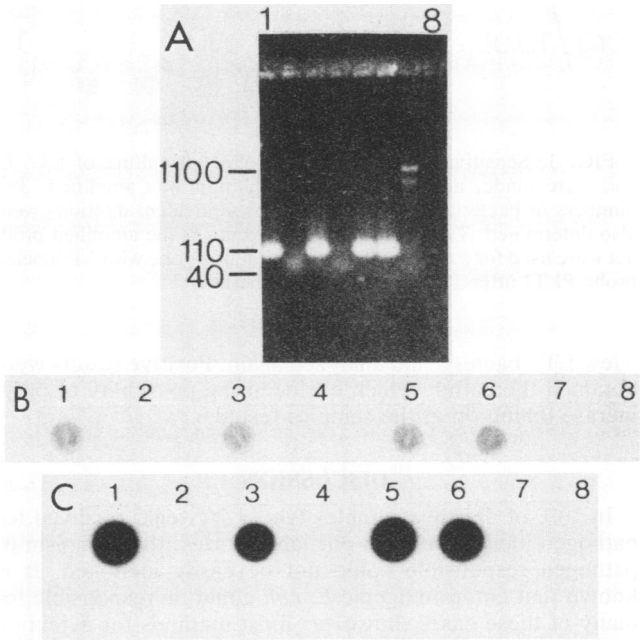

FIG. 2. Specificity. Control bacterial strains were directly amplified from saline solutions with primers LT51 and LT31. Twenty-microliter samples of the PCR product were used for dot blot hybridization or agarose gel electrophoresis. Detection of the expected 110-bp product was done with ethidium bromide (A) or hybridization with alkaline phosphatase-labeled probe PLT2 (B) and ^{32}P-labeled probe PLT1 (C). Filter C was autoradiographed at −70°C for 2 h. The samples were LT$^+$ *E. coli* (lane 1); saline (lane 2); LT$^+$ *E. coli* (lane 3); ST$^+$ *E. coli* (lane 4); LT$^+$ *E. coli* (lane 5); a mixture of LT$^+$ *E. coli*, *Shigella dysenteriae*, *Salmonella typhimurium*, and *K. pneumoniae* (lane 6); *Shigella dysenteriae* (lane 7); and *Salmonella typhimurium* (lane 8). The numbers to the left of panel A indicate molecular sizes in base pairs. A 1.7% agarose gel was used.

Sensitivity. Serial dilutions of a 6-h culture of LT enterotoxin-producing *E. coli* were made, and 10 μl of each dilution was amplified. Dot blot detection was done with ^{32}P-labeled probe PLT1 as described in Materials and Methods. The numbers of bacteria in 10-μl volumes of the equivalent dilutions were also determined. As little as 1 bacterium in 10 μl could be detected by using a ^{32}P-labeled probe after 10 h of exposure of the filter (Fig. 3). No signal was obtained after 10 h of exposure of a negative control sample (data not shown). Alkaline phosphatase-labeled probe PLT2 was 10% less sensitive (data not shown). The electrophoresis detection system required a minimum of 100 bacteria in 10 μl. The same sensitivities were obtained when a mixed culture of LT$^+$ and LT$^-$ strains was used.

Analysis of clinical isolates. Clinical isolates were analyzed by using the electrophoresis system, and 12 of 132 samples were positive for the enterotoxin plasmid. A sample result is shown in Fig. 4. The negative samples were all spiked with

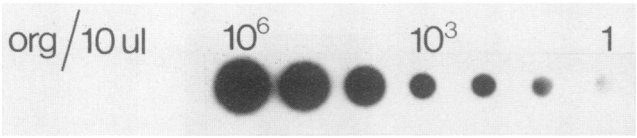

FIG. 3. Sensitivity. Serial dilutions of a 6-h culture of LT^+ *E. coli* were made, and 10 μl of each dilution was amplified. The numbers of bacteria (org) in 10 μl of the equivalent dilutions were also determined. Twenty-microliter portions of the amplified product were used for a dot blot, and detection was done with ^{32}P-labeled probe PLT1 after 10 h of exposure of the filter.

a few LT^+ bacteria and analyzed again. Positive results were obtained thereafter, which excluded the possibility of polymerase inhibition in the samples tested.

DISCUSSION

In 10% of diarrhea samples (about 20/week) received for pathogen identification in our laboratories, the organism or pathogen responsible could not be easily identified. It is known that enterotoxigenic *E. coli* could be responsible for many of these cases; however, most methods for detection of this organism are unsatisfactory, being complex, time-consuming, and insensitive (9, 14, 17). In addition, serotyping can give false-negative results when strains of *E. coli* regarded as nonpathogenic (i.e., normal flora) may become pathogenic owing to conjugation of the gene (plasmid) from one strain to another. These methods also require large numbers of organisms for detection.

For these reasons, a number of reports have appeared recently detailing improved methods for detection (6, 10, 11, 13, 16). All of these methods have some inherent problems or disadvantages, and we therefore felt that there was room for improvement. We have concentrated on establishing an easy, quick, and inexpensive screening method for large numbers of clinical samples of LT enterotoxin-producing *E. coli* using PCR amplification of DNA. A similar method for detection of LT toxin-producing *E. coli* has been used by others (3, 12); however, we have selected a set of primers and a target area different from those of others (12), since careful selection of the area of the gene to be used is critical to ensure a high degree of specificity. We noted that human LT or pig *E. coli* enterotoxin is structurally and functionally similar to enterotoxin from *Vibrio cholerae* (18). In addition, minor variations in the *E. coli* enterotoxin gene have been recorded from a less conserved area of this gene (19). We therefore selected primers and a region of the enterotoxin gene such that a highly conservative region of the LT enterotoxin gene would be amplified, thus avoiding problems possible in other systems (12).

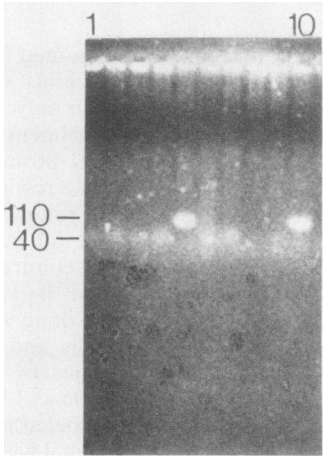

FIG. 4. Detection of LT toxin-producing *E. coli* in clinical isolates. Diarrhea samples (mainly from infants) were routinely screened for salmonellae, shigellae, or parasite infection. In samples in which the pathogen could not be detected, a scrape of the mixed colony growth from the MacConkey plate was directly amplified with primers LT51 and LT31 as described in Materials and Methods. Twenty-microliter samples of the amplified product were used for gel electrophoresis and detection with ethidium bromide. The sample in lane 1 contained saline instead of template DNA. The only LT^+ samples were those in lanes 5 and 10. The numbers on the left indicate molecular sizes in base pairs. A 1.7% agarose gel was used.

We obtained a high degree of sensitivity and specificity. The conditions reported were empirically determined to give optimal results. We found that preselection of bacteria on plates from stool samples is important for a number of reasons. (i) This step is not an extra step, as it is routinely done to screen for salmonellae and shigellae. (ii) There have been a number of reports which show that *Taq* polymerase can be inhibited in the presence of raw biological samples (e.g., stool and urine) (1, 12). (iii) We did not have to isolate DNA material from the samples before amplification, as was done in other PCR-dependent systems (3, 12). Prior extraction of DNA is a very time-consuming and expensive step and does not lend itself easily to testing of large numbers of samples.

The specificity and sensitivity of our method are possibly better than those obtained by others (7, 12). In our system, for example, we did not experience inhibition of reaction with larger numbers of bacteria, as reported elsewhere (12). This could be a function of reaction conditions, since we used three temperature cycles and 0.5 U of polymerase, as opposed to the two cycles and 5 U described elsewhere (12). This also had a favorable influence on the final cost per assay.

While dot blot hybridization to a radiolabeled third oligonucleotide, as reported here, can enhance the degree of certainty that one is detecting a specific product, it is not the method of choice for a routine diagnostic laboratory. For this reason, we did this hybridization with a nonradioactive probe with very little loss in sensitivity. This method has the added advantage that the labeled probe is stable and can be stored for many months for use as required.

However, our results suggest that even this is unnecessary and that simple agarose gel electrophoresis on a minigel is sufficient for routine diagnosis of this pathogen. This method is quick, nonhazardous, and cheap, and although this system is 100 times less sensitive than the ^{32}P-labeled probe detection system, we detected no positive signals in negative gel samples. We suggest that to produce clinical symptoms, sufficiently large numbers of LT^+ *E. coli* organisms are present for safe use of the electrophoresis technique and that the extra sensitivity given by hybridization is not necessary for clinical testing. These extra systems may prove useful in testing the environment for LT^+ *E. coli*, however, since fewer organisms are encountered.

Since we selected our primers with care, we experienced no synthesis of nonspecific products in the size range of the enterotoxin gene product. When this did occur, it was traced to contamination of reagents. This is perhaps the most limiting factor in the use of the PCR technique, and all possible precautions must be taken to avoid this problem (5,

8, 15). *Shigella dysenteriae* often produces faint higher-molecular-weight DNA fragments, which are clearly different from the LT toxin product. A nonspecific low-molecular-weight product, which may be due to a primer dimer artifact, is also present, as in most other PCR systems (5).

Although we report a method for detection of the LT enterotoxin gene only, it should be possible to apply this approach to the detection of other enterotoxin genes (e.g., ST toxin) or pathogens, since relevant nucleotide sequence data are rapidly becoming available. An attempt has recently been made to perform multigene amplification, but amplification of two or three genes simultaneously was less efficient than amplification of individual genes. Although the amplified product can be detected with a corresponding labeled probe, this procedure involves complex extra extraction and hybridization steps (3).

The results presented in this report show that our system is highly sensitive and specific for detection of LT toxin-producing *E. coli* and that about 9% of patients with nondiagnosed diarrhea in Tygerberg Hospital were infected with this pathogen.

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